An intergenic non-coding RNA targets and regulates the ribosome in *H. volcanii*.

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Aim of the project

As translation is the final step in gene expression it is particularly important to understand the processes involved in translation regulation. It was shown in the last years that a class of RNA, the non-protein-coding RNAs (ncRNAs), is involved in regulation of gene expression via various mechanisms. Herein included is the prominent example of gene silencing caused by micro RNAs (miRNAs) and small interfering RNAs (siRNAs). Almost all of these ncRNA discovered so far target the mRNA in order to modulate protein biosynthesis, this is rather unexpected considering the crucial role of the ribosome during gene expression. However, recent data from our laboratory showed that there is a new class of RNAs among the well-studied ncRNAs that target the ribosome itself . These so called ribosome-associated ncRNAs (rancRNAs) have an impact on translation regulation, mainly by interfering / modulating the rate of protein biosynthesis (1,2). The main goal of this project is to identify and describe novel potential regulatory rancRNAs in *H. volcanii* with the focus on intergenic candidates.

Model organism

Haloferax volcanii belongs to the lineage of Euryarchaeota, which may be the most ancient that exists on earth. It was first isolated from the Dead Sea and requires high salt concentrations (2-4 M NaCl) and 42° C for growth.
H. volcanii is easy to cultivate, the genome is sequenced, transcriptomics and proteomics are established and methods for genetic manipulation are available, thus making it an ideal archaeal model organism.

Results 1: ribosome associated ncRNAs (rancRNAs) in *H.volcanii*

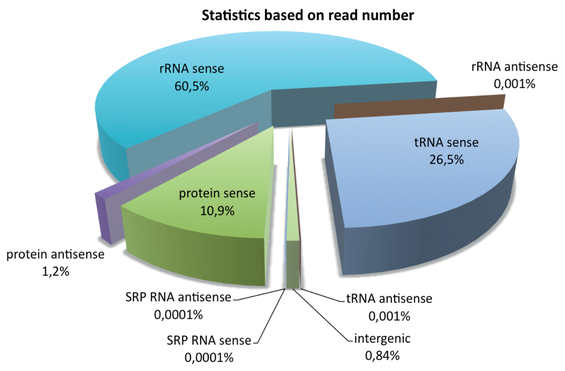


Fig1: Deep sequencing results

In the course of studying the ncRNA interactome of the archaeal ribosome, we have constructed a specialized cDNA library from small RNAs (sized 20-500 nt) that co-purify with ribosomes from *H. volcanii* under different environmental stress conditions (3). Subsequent deep sequencing yielded 73.5 Mio raw reads which were analyzed using the APART pipeline, ending up with 6,250 putative rancRNA candidates.

Results 2: RNA s194 an intergenic rancRNA

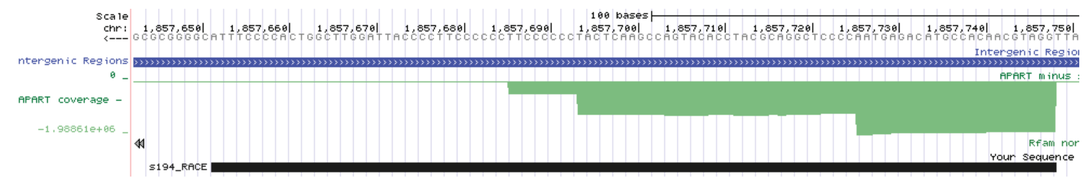


Fig2: Screen shot of UCSC genome browser showing RNA candidate s194.

The rancRNA s194 was the most abundant rancRNA among the intergenic rancRNAs found in our library. Due to oligoG adaptors used for cDNA library preparation the full length could not be detected. Therefore 3' RACE was performed and the full length of s194 was determined to be 97 nt (see black bar in Fig:2).

Results 3: rancRNA s194 associates with ribosomes *in vivo* and *in vitro*

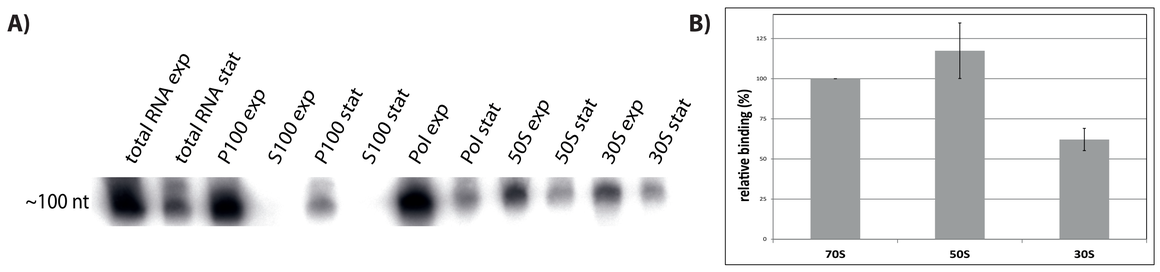


Fig3: A) Northern blot of rancRNA s194 from cells grown to either exponential or stationary growth phase. First 2 lanes total RNA, lanes 3 and 5 RNA extracted from pellet of 100000 x g centrifugation containing ribosomes (P100), lanes 4 and 6 RNA extracted from the supernatant of 100000 x g centrifugation (S100) and lanes 7-12 RNA isolated from different ribosomal particles. B) Filter binding analysis: binding of radiactive labeled *in vitro* transcribed s194 RNA to different ribosomal particles isolated from *H. volcanii* s194 knock-out strain. C) Binding stoichiometry of s194 RNA. Increasing amounts of radio-labeled in vitro transcribed s194 RNA was incubated with 5 pmol 50S isolated from wild type *H. volcanii* cells.

Results 4: Growth studies with rancRNA s194 knock out strain

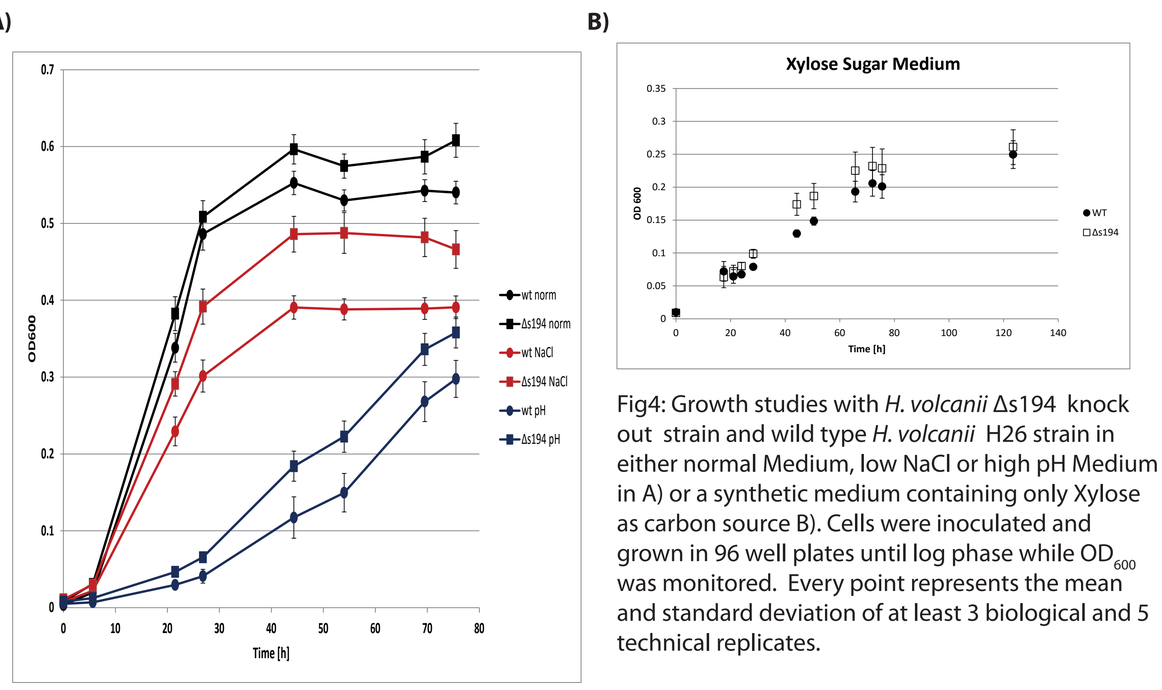
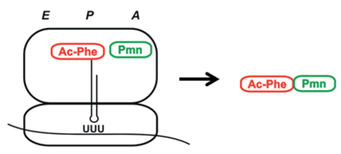


Fig4: Growth studies with *H. volcanii* Δs194 knock out strain and wild type *H. volcanii* H26 strain in either normal Medium, low NaCl or high pH Medium in A) or a synthetic medium containing only Xylose as carbon source B). Cells were inoculated and grown in 96 well plates until log phase while OD_{600} was monitored. Every point represents the mean and standard deviation of at least 3 biological and 5 technical replicates.

Results 5: RancRNA s194 inhibits peptidyl transferase reaction *in vitro*



Puromycin reaction with *H. vol* 70S

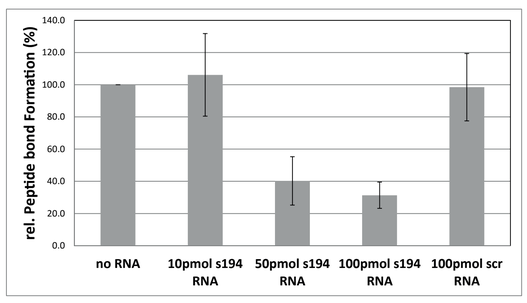


Fig5: Peptidyl transferase reaction catalyzed by 10 pmol of *H. volcanii* ribosomes isolated from a rancRNA s194 knock out strain. Increasing concentrations of in vitro transcribed s194 RNA were added as well as a scrambled control. In average 48% of the N-acetly-3H-Phe-tRNA initially used could be transferred to puromycin in the no RNA control. The values given represent the mean and standard of 3 experiments.

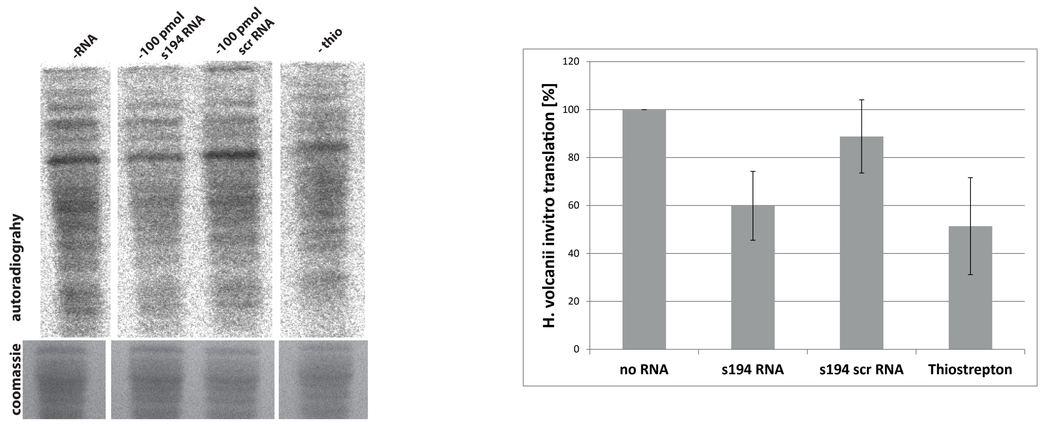


Fig6: *in vitro* translation in *H. volcanii*. To cell extract of *H. volcanii*, ^{35}S-methionine was added to monitor newly synthesized proteins in presence or absence of synthetic RNA or the antibiotic thiostrepton. The radiolabeled proteins were then visualized by SDS PAGE and subsequent autoradiography. The Coomassie-stained gel serves as a loading control. The values given represent the mean and standard of 3 experiments.

Results 6: RancRNA s194 inhibits protein production *in vivo*

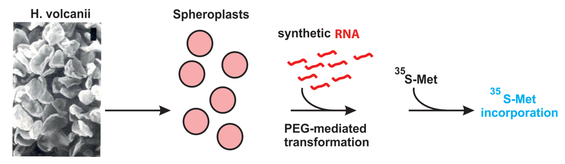
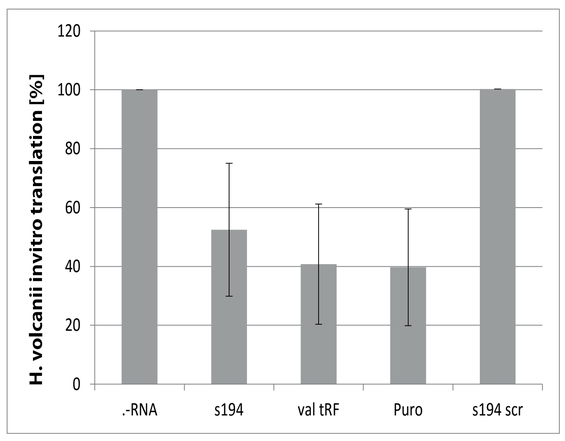
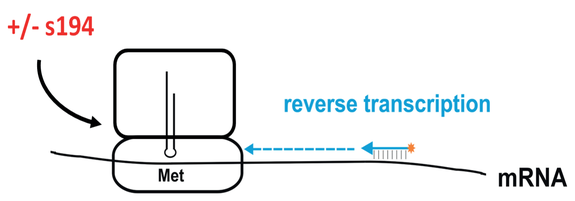


Fig7: metabolic labeling. H. volcanii cells were transformed with indicated RNA via PEG600 and metabolic activity was measured from this time point on with addition of ^{35}S-methionine. Proteins were TCA precipitated and filtered and the amount of newly synthesized proteins was measured via liquid scintillation counting. As controll a tRNA fragment val-tRF RNA was added which is known to inhibit protein translation in H. volcanii. The values given represent the mean and standard of 3 experiments (except s194 scr only 1 experiment).



Results 7: Toeprinting assay shows that s194 has no effect on initiation of translation.



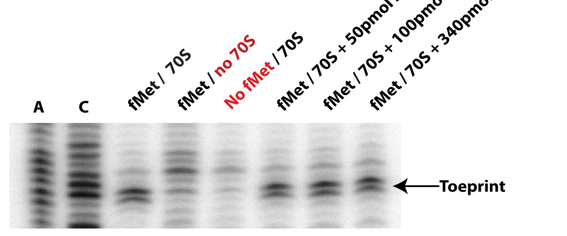


Fig8: Toeprinting assay. Lanes 1+2: sequencing lanes. Lane 3: Hfx wild type 70S and deacylated tRNAfMet form an initiation complex on an mRNA. Lanes 4+5: No initiation complex formed when either 70S or fMet is missing. Lanes 6-8: increasing concentrations of *in vitro* transcribed s194 RNA do not influence formation of the tranlsation initiation complex as there is no change in the toeprint signal.

(1) A. Pircher, K. Bakowska-Zywicka, L. Schneider, M. Zywicki, N. Polacek, An mRNA-derived noncoding RNA targets and regulates the ribosome. Molecular cell 54, 147 (Apr 10, 2014).

(2) A. Pircher, J. Gebetsberger, N. Polacek, Ribosome-associated ncRNAs: an emerging class of translation regulators. RNA biology 11, 1335 (2014).

(3) J. Gebetsberger, M. Zywicki, A. Kunzi, N. Polacek, tRNA-derived fragments target the ribosome and function as regulatory non-coding RNA in Haloferax volcanii. Archaea 2012, 260909 (2012).

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